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以癌溶性病毒作肝原位癌的基因治療(第 2 年)
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前言

Strategies to increase antitumor efficacy of oncolytic adenoviruses are actively investigated. We have previously shown that E1B-55 kD-deleted adenovirus, designated Ad5WS1, has therapeutic potential for treating hepatocellular carcinoma (HCC). To achieve HCC-restricted replication of oncolytic adenovirus, we generated Ad5WS2, an E1B-55 kD-deleted adenovirus with its E1A gene driven by the liver-specific transthyretin (TTR) promoter.

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研究目的

The aims of this study are to exploit tumor selective and liver-specific replication-selective oncolytic adenoviruses, Ad5WS2 in combination with chemotherapy for the treatment of HCC. Four specific aims were focused in the study: (1) Test the expression of the TTR gene in clinical sample of HCC patients, (2) Treatment of multifocal HCC in the liver of immune-competent mice by systemic injection of Ad5WS2 via tail vein, (3) Treatment of malignant ascites associated HCC of immune-competent mice by intraperitoneal injection of Ad5WS2, (4) Evaluation of tissue toxicity and immune response after virus administration. Our results showed that Ad5WS2 could replicate within tumor cells where transthyretin gene was expressed. Mouse transthyretin promoter was active in murine and human HCC cells, but relatively quiescent in cells of non-liver origin. Ad5WS2 caused severe cytolytic effect on HCC cells, but was much attenuated in non-HCC cells. Peritoneal administration of Ad5WS2 into mice bearing liver tumors grown in ascites resulted in enhanced survival. In an orthotopic HCC model, Ad5WS2, when systemically administered, exerted higher antitumor effects than Ad5WS1. Lack of viral replication in normal organs and minimal hepatic toxicity was noted after Ad5WS2 treatment. Furthermore, the antitumor effect of Ad5WS2 could be enhanced when combined with chemotherapeutic agent cisplatin in the ascites tumor model.

文獻探討

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide.[1] Owing to its heterogeneity and ease of tendency to develop multidrug resistance phenotypes, HCC is refractory to conventional treatment.[2] The use of conditionally replicating viruses, including adenovirus, has offered a promising platform for selective cancer treatment.

ONYX-015, which is one of the most studied oncolytic adenoviruses, can selectively replicate in tumor cells while sparing normal cells. Several clinical trials, such as head and neck cancer, ovarian cancer, and pancreatic cancer, have supported the therapeutic use of ONYX-015 for the treatment of solid tumors.[3-5] Clinical trials in patients with primary liver cancers or gastrointestinal carcinoma metastatic to liver have also been reported.[6-8] Despite such encouraging results, using oncolytic adenoviruses alone for cancer therapy were so far disappointing.[9] One of the major barriers to the successful clinical translation is the difficulty in successful targeting and quantitative infection of relevant cell types. To increase viral oncolysis, numerous efforts have been made to improve the therapeutic efficacy of oncolytic adenoviruses by expressing transgenes[10-12] or modifying viral fibers to extend viral tropism.[13] To target oncolytic adenoviruses to liver tumors, E1B-55 kD-deleted adenovirus driven by the albumin or α-fetoprotein promoter has been utilized to control the expression of early viral genes, such as E1A, to achieve tumor-specific adenoviral replication in HCC cells.[14-17]. Transthyretin is a thyroid hormone transport protein that is secreted into the serum by hepatocytes and into the cerebrospinal fluid by choroid plexus.[18] A 200-nucleotide sequence from the 5'end to the cap site is highly conserved between mouse and human genes. Sequence alignment indicated an 80% homology at the protein level between mouse and human genes.[19] Deletion analysis identified a crucial element in the 5'-flanking regulatory region for specific expression in mouse liver and human hepatoma cell lines.[20, 21] The transthyretin promoter has been used to study the function of trangenes in the liver from transgenic mice.[22] These findings suggest that transthyretin may serve as a specific marker for targeting liver tumors.

In this study, we constructed a liver-specific E1B-55 kD-deleted adenovirus, designated Ad5WS2, under the control of the transthyretin promoter. Our results show that Ad5WS2 selectively replicates and hence lysed HCC cells. Furthermore, Ad5WS2 exhibited high antitumor activity via systemic administration in an orthotopic syngeneic HCC model. The antitumor effect of Ad5WS2 was in synergy with cisplatin in mice bearing liver tumors accompanied with malignant ascites. Therefore, Ad5WS2 represents a potentially applicable anticancer agent for the treatment of primary and metastatic HCC.

研究方法

Cell lines and mice. ML-1 (mouse HCC)[23], CT-26 (mouse colon cancer), NIH-3T3 (mouse fibroblast), LL2 (mouse Lewis lung carcinoma), SK-Hep1, Hep3B (human HCC)[24, 25], Chang liver (non-malignant human hepatocytes)[25], HT1376 (human bladder cancer)[26], and 293 (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 50 μg/ml gentamicin at 37℃. Female BALB/c mice and C57BL/6 mice at the age of 7 weeks were obtained from the Laboratory Animal Center of the National Cheng Kung University. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University.

Construction of Ad5WS2 oncolytic adenovirus. Ad5WS1, an E1B-55 kD-deleted adenovirus, has been described previously.[27] The 3' region of the E1A promoter in the backbone of Ad5WS1 was interrupted by the mouse tranthyretin promoter, resulting in generation of Ad5WS2. To insert a multiple cloning site at nucleotide 525 of adenovirus genome, the adenovirus region from bases 343 to 1003 was generated by the polymerase chain reaction (PCR) using pAd5WS1 as the template. The resulting 660-bp fragment restricted with *Not*I and *Sac*I was cloned into the pBluescript II SK(+/-) to generate pBSII-0.7. The adenovirus E1 region from bases 525 to 2270 was also generated by PCR using pAd5WS1 as the template. The resulting 1500-bp fragment restricted with *Xho*I and *Kpn*I was cloned into the *Xho*I and *Kpn*I sites of pBSII-0.7, resulting in pBSII-0.7-1.5. The 2.7 kb-fragment of pBSII-0.7-1.5 containing multiple cloning sites and part of E1 sequence was digested with *Not*I and *Kpn*I and cloned into pAd5WS1, yielding pAd5YS.

The 330-bp fragment of mouse transthyretin promoter was obtained by PCR using chromosome DNA from mouse embryonic fibroblasts as the template and cloned into the *Hin*dIII site of pGL-Basic (Promega, Madison, WI), yielding pGL-TTRp, a luciferase reporter plasmid driven by the transthyretin promoter. Meanwhile, the transthyretin promoter region was excised from pGL-TTRp and cloned into the *Hin*dIII site of pAd5YS to generate pTTRp/YS. The 3000-bp fragment of pTTRp/YS was digested with *Bsr*GI and *Mun*I and subcloned into an adenoviral shuttle vector pShuttle[28] to generate pTTRp/YS/PS, a shuttle vector for generating Ad5WS2. Ad5WS2 virus was then produced using the AdEasy system as previously described.[28]

Analysis of transthyretin promoter activity. The transcriptional activity of the transthyretin promoter was assessed as previously described.[27] Briefly, various cells were cotransfected with 2 μg of pGL-TTRp containing a luciferase expression

cassette driven by mouse transthyretin promoter and 1 μg of pTCYLacZ, a β-galatosidase reporter plasmid driven by the β-actin promoter[29] by lipofectin. Cell lysates were harvested 48 h after transfection and their luciferase activities were determined.

Immunoblot, immunohistochemical and immunofluorescence analysis. Total cell lysates were prepared as previously described.[27] Lysates prepared from liver tissues of HCC patients or mice were homogenized in PBS containing protease inhibitor cocktail (Pierce, Rockford, IL). These samples were subjected to immunoblot analysis using antibodies against human transthyretin (DAKO, Carpinteria, CA), adenovirus E1A (M73, Santa Cruz Biotechnology, Santa Cruz, CA), adenovirus hexon (abcam, Cambridge, UK), adenovirus fiber (4D2, abcam), and β-actin (AC-15, Sigma, St. Louis, MI).

Tumors nodules as well as liver and spleen tissues were collected and snap frozen. Cryostate sections (5 μm) were prepared and incubated with antibody against human transthyretin (DAKO) or adenovirus E1A (clones 20/11 and 2/6, Chemicon, Temecula, CA), followed by detection with the DAKO LSAB 2 System (DAKO) according to the manufacturer's instructions.

Cells were fixed in paraformaldehyde, permeabilized with cold methanol/acetone (90:10 v/v), incubated with antibody against human transthyretin (DAKO) and adenovirus fiber (4D2, abcam), and subsequently incubated with fluorescein-conjugated goat anti-rabbit IgG (KPL) and Texas Red-conjugated goat anti-mouse IgG (KPL). Nuclei were stained with 50 μg/ml of DAPI (4', 6-diamidino-2-phenylindole dihydrochloride).

Assay of cell viability and viral replication. The viable cell numbers were determined in cells seeded in 6-well plates after 3 days of virus infection at a multiplicity of infection (MOI) of 2 by trypan blue exclusion. Viral replication was assessed as previously described.[27] Briefly, cells were infected with either Ad5WS2 or wild-type adenovirus type 5 (Ad5WT) (MOI=10). Viruses harvested from both the supernatant and cell lysate at 53 h postinfection were pooled and titered on 293 cells for 50% tissue culture infective dose ($TCID_{50}$) calculated by the Reed-Muench Method. The relative virus replication efficiency was calculated by the equation as described previously.[30] A colorimetric assay using WST-1 (Dojindo Labs, Tokyo, Japan) was used to assess cell viability. ML-1 cells were treated with Ad5WS2 (MOI=50) and cisplatin (10 mM). Four days after treatment, 100 μl of WST-1 (Dojindo Laboratories, Tokyo, Japan) was added to each well and incubated at 37℃ for 1 h. The absorbance at 450 nm that stands for cell growth was measured with the reference wavelength at 595 nm.

***In vivo* antitumor efficacy.** ML-1 and LL2 cells (1×10^6) were inoculated subcutaneously (s.c.) into the right flank of BALB/c and C57BL/6 mice at day 0, respectively. Twelve days after tumor cell inoculation, eight mice were randomly allocated to various treatment groups. Mice were treated intratumorally (i.t.) with Ad5WS2 or Ad5WS1 (2×10^7 PFU, plaque-forming unit) at different day. Tumors were measured once a week and the tumor volume was calculated as: (length of tumor) $\times$ (width of tumor)^2 $\times$ 0.45. To establish ascites tumor models, ML-1 and LL2 cells (2×10^6) were inoculated intraperitoneally (i.p.) into BALB/c and C57BL/6 mice at day 0, respectively. Three days after tumor cell inoculation, seven mice were randomly allocated to various treatment groups. Mice were injected i.p. with Ad5WS2 or Ad5WS1 (5×10^7 PFU) at different day. To establish an orthotopic liver tumor model, ML-1 cells (2×10^5) were inoculated into the left liver lobe of BALB/c mice at day 0 as previously described.[31] Groups of 6-7 mice bearing orthotopic ML-1 tumors were injected intravenously (i.v.) with Ad5WS2 or Ad5WS1 (2×10^7 PFU) at different day. For combination therapy, mice that had been inoculated with ML-1 cells (2×10^6) at day 0 were injected i.p. alternatively with four doses (5×10^7 PFU/dose) of Ad5WS2 and four doses of cisplatin (4 mg/kg) at different day. All mice were monitored for survival after tumor inoculation.

Determination of serum ALT (alanine aminotransferase) level. Groups of 4 mice were inoculated with ML-1 cells (2×10^6) at day 0 and then injected i.p. with Ad5WS1 and Ad5WS2 (5×10^7 $TCID_{50}$) at day 3. Mouse sera collected 19 h, as well as 3 days after virus injection were measured for their ALT concentrations using vitros DT chemistry system (Johnson & Johnson, Rochester, NY).

Statistical analysis. The survival analysis was done using the Kaplan-Meier survival curve and the log-rank test. Other statistical differences were assessed with Student's *t* test. $P < 0.05$ is regarded statistically significant.

結果與討論

Liver specificity of transthyretin promoter. To ensure transthyretin expression in human liver tissues, we tested the expression of transthyretin protein in 6 tumor and 4 non-tumor tissues, which included 3 paired samples, from HCC patients (Fig. 1a). The levels of tranthyretin expression in HCC tumor tissues were comparable or even 1.5- to 2-fold higher than those from normal liver tissues determined by densitometric analysis. HCC cells appeared to produce more transthyretin than normal liver cells (P=0.04) (Supporting information Fig.S1.). We next examined the transcriptional activity of the transthyretin promoter in cell from liver and non-liver origin using a luciferase reporter assay. The cell lines used included human HCC (Hep3B and SK-Hep1), human bladder cancer (HT1376), human non-malignant hepatocytes (Chang liver), murine HCC (ML-1), and murine lung cancer (LL2) cells. As shown in Fig. 1b, the transthyretin promoter was active in liver cells, with highest activity in Hep3B cells and moderate activity in Chang liver, ML-1, and SK-Hep1 cells. Therefore, the mouse tranthyretin promoter may also use human transcription machinery as efficiently as using its mouse counterpart. However, HT1376 and LL2 cells exhibited very low transthyretin promoter activity. To ensure tissue specificity of Ad5WS2 replication, we examined the expression of late viral protein in three cell lines originated from BALB/c mice. As shown in Fig. 2, fiber protein was expressed in ML-1 cells where *transthyretin* gene was active, whereas the virus was scarcely detected in CT-26 (colon cancer) and NIH-3T3 cells (normal fibroblast) with low expression of transthyretin (Supporting information Fig.S2., Fig.S3.). Therefore, among different tissues originated from the same host, adenovirus could only replicate in tumor cells where *transthyretin* gene was expressed.

Replication specificity of Ad5WS2 in HCC cells. We have previously shown the antitumor efficacy of Ad5WS1 in HCC and bladder cancer in animal models.[27, 32] We next investigated whether Ad5WS2 selectively induced cytolytic effect in HCC cells (Supporting information Fig.S4.). The viability of Hep3B or SK-Hep1 cells infected with Ad5WS2 was lower than that infected with Ad5WS1, whereas cell survival was decreased to a similar extent in Ad5WS2-infected ML-1 cells as in their Ad5WS1-infected counterparts (Fig. 3a). Notably, the viability of Ad5WS2- or Ad5WS1-infected Chang liver cells did not differ significantly from that of their mock-infected counterparts. By contrast, whereas Ad5WS1 induced significant cell death in HT1376 and LL2 cells, Ad5WS2 did not cause significant cell death in these cells. To test the replication of Ad5WS2 in cell lines from different origin, the level of Ad5WS2 produced at 53 h postinfection was determined and normalized against that of Ad5WT produced in the same cell line. The absolute virus

titers of Ad5WS2 produced in ML-1, Hep3B and SK-Hep1 cells were 5×10^7 $TCID_{50}$/ml, 1.7×10^8 $TCID_{50}$/ml and 1×10^7 $TCID_{50}$/ml, respectively. Fig. 3b shows that the relative viral production was highest in Hep3B cells, followed by SK-Hep1 and ML-1 cells. By contrast, Ad5WS2 production reached only 0.41% and 16% of Ad5WT in HT1376 and LL2 cells, respectively, which exhibited a relatively resistant phenotype to Ad5WS2 infection. Notably, Chang liver cells, which were immortalized human hepatocytes with moderate transthyretin promoter activity, were also resistant to Ad5WS2 infection (2.35% relative to Ad5WT). Immunoblot analysis revealed that E1A protein was identified in both Hep3B and ML-1 cells albeit to a low level of protein in Chang liver cells 36 h after Ad5WS2 infection (Fig. 3c). Hexon and fiber proteins, were also detected not only in human Hep3B HCC cells but also in mouse ML-1 HCC cells following Ad5WS2 infection 48 h postinfection (Fig. 3d). However, hexon protein was undetectable, and fiber proteins were present at a negligible level in Ad5WS2-infected Chang liver cells. Taken together, these results suggest that replication of Ad5WS2 was restricted to HCC cells.

Antitumor efficacy of Ad5WS2 in mice bearing ML-1 tumors. Giving that immunocompetent mouse tumor models are more relevant to clinical applications and that murine ML-1 cells are permissive for Ad5WS2 replication, we employed the ML-1 tumor model for animal studies. Tumor growth was first evaluated in ML-1 tumor-bearing BALB/c mice and LL2 lung tumor-bearing C57BL/6 mice injected i.t. with Ad5WS2 or Ad5WS1. The growth of ML-1 tumors was significantly retarded in Ad5WS2-treated (P=0.007) or Ad5WS1-treated (P=0.002) mice compared with that in PBS-treated mice (Fig. 4a). However, in LL2 tumor-bearing mice, whereas Ad5WS1 significantly retarded tumor growth (P=0.00007), Ad5WS2 only exhibited marginal antitumor effect compared with PBS treatment (P=0.09) (Fig. 4b). We also used replication-defective AdLacZ to infect ML-1 and LL2 cells and confirmed similar susceptibility of the two cell lines to adenoviral infection (data not shown). In consistent with *in vitro* data (Fig. 3), these results indicate that Ad5WS2 was cytolytic to tumor cells in which transthyretin promoter was active, which resulted in suppressing tumor growth. We next used ML-1 ascites tumor model to test whether Ad5WS2 could systemically target HCC cells *in vivo*. We treated mice bearing ML-1 ascites tumors with Ad5WS2 or Ad5WS1. In tumor sections from animals treated with Ad5WS2 or Ad5WS1, hexon protein-positive tumor cells were detected in peritoneal tumor nodules, suggesting that active replication of either Ad5WS2 or Ad5WS1 may have occurred (Fig. 4c). In a separate experiment, we treated mice bearing ML-1 or LL2 ascites tumors with Ad5WS2 or Ad5WS1. Fig. 4d shows that in mice bearing ML-1 ascites tumors, Ad5WS2 treatment (P=0.0034) significantly prolonged the survival time, whereas Ad5WS1 treatment (P=0.0572) only marginally

enhanced mouse survival compared with PBS treatment. The median survival in Ad5WS2-treated group is 36 days, longer than Ad5WS1 (31 days) and PBS-treated ones (30 days). Nevertheless, as shown in Fig. 4e, Ad5WS2 treatment failed to enhance the survival time of the mice bearing LL2 ascites tumors compared with PBS treatment (P=0.934). Collectively, these results suggest more potent inhibition of liver tumors by Ad5WS2 than by Ad5WS1 in mice. As orthotopic liver tumors more closely resemble the biological characteristics of the human tumor, we next used ML-1 cells grown in the liver of their syngeneic hosts to evaluate the bio-distribution of Ad5WS2 after i.v. injection. Immunoblot analysis revealed that virus fiber protein was only detected in liver tumors but not normal liver and spleen tissues (Fig. 5a). Fig. 5b shows that Ad5WS2-treated mice exhibited enhanced survival compared to PBS-treated (P=0.008) or Ad5WS1-treated (P=0.0257) animals. Notably, Ad5WS2 was more efficacious than Ad5WS1 in prolonging the survival time and increasing the survival rate in mice bearing orthotopic liver tumors (P=0.0257). Importantly, three of seven Ad5WS2-treated mice remained alive at the time of sacrifice. In a separate experiment, we i.v. treated mice bearing ML-1 orthotopic tumors with Ad5WS2 or Ad5WS1. In contrast to Ad5WS1-treated animals, virus protein expression was restricted only in Ad5WS2-treated tumor section indicating more specific spreading of Ad5WS2 (Fig. 5c). A mild transient elevation of ALT levels was noted at 19 h postinfection compared to the control counterpart (P=0.016) and back to the normal range at 3 days postinfection (Fig. 5d). However, Ad5WS1-treated mice exhibited higher ALT levels at 19 h and 3 days after administration compared to the Ad5WS2-treated ones (P=0.032, P=0.0006, respectively). There was a positive correlation between the viral load in livers and serum ALT levels, suggesting that the specificity of Ad5WS2 may contribute to decreased hepatic toxicity through systemic administration.

Additive antitumor efficacy of Ad5WS2 in the combination of cisplatin on mice bearing ascites ML-1 tumors. To further enhance the antitumor effect of Ad5WS2, we used the combination therapy of Ad5WS2 with cisplatin. Cells treated with cisplatin given only moderate cytolytic effect (Fig. 6a, P=0.037). However, the combination treatment of Ad5WS2 plus cisplatin significantly reduced tumor cell growth (P=0.00015). In mice bearing ML-1 ascites tumors, either Ad5WS2 (P=0.0066) or cisplatin (P=0.0004) alone prolonged the survival compared with PBS treatment (Fig. 6b). Of note, combination therapy significantly increased the survival time of the animals compared with either Ad5WS2 (P=0.0002) or cisplatin treatment alone (P=0.0011). Collectively, these results demonstrate that systemic administration of Ad5WS2, in particular combined with cisplatin, prolonged the survival of the animals bearing liver tumors grown

orthotopically or in ascites.

In this study, we exploited Ad5WS2, an E1B-55 kD-deleted adenovirus driven by the transthyretin promoter for HCC treatment. Ad5WS2 was derived from Ad5WS1,[27] which is similar to ONYX-015 frequently used in gene therapy,[33] by replacing its internal E1A promoter with transthyretin promoter. This is the first time that transthyretin promoter was used to construct a liver tumor-specific oncolytic virus. Although transthyretin was also consistently expressed in brain, the endogenous brain expression was ~10-20% of that observed in the liver. Yan *et al.* reported that in transgenic mice carrying 1-2 copies of the regulatory regions of gene, which was sufficient for high level hepatic expression, showed very low, nearly undetectable transgene expression in the brain.[18] No transgene expression was detected in spleen, salivary gland, heart, lung, intestine, and striated muscle. This regulatory region contains several binding sites for hepatocyte nuclear factors (HNFs). It is believed that HNF-1, HNF-3, and HNF-4 were highly enriched in liver but lacking in the choroids plexus tissues conferring to the different gene expression in tissues.[34] The same regulatory region (330 bp) was used as the promoter of Ad5WS2. Therefore, the damage to the choroids plexus could be reduced to minimum. Moreover, Ad5WS2 also harbored deletion in the E1B-55 kD protein. Giving that the functions of E1B-55 kD include RNA export, host protein shutoff, as well as p53 degradation, O'shea *et al.* demonstrated that the oncolytic selectivity of E1B-55kD-deleted adenovirus was determined by a property of tumor cells to efficiently export late viral RNA in the absence of E1B-55kD, a propensity not shared by normal cells.[35] Thus, permissive liver tumor cells could support Ad5WS2 replication by providing the late function of E1B-55kD. One of viral late RNAs, fiber, could be detected in Hep3B cells, but not in the Chang liver cells after Ad5WS2 infection (Supporting information Fig.S5). Moreover, we had also observed significantly reduced fiber expression, and a distinct lack of hexon expression at 48 hours postinfection with Ad5WS2 in Chang liver cells compared to the Hep3B and ML-1 cells. As non-malignant human liver cells, although Chang liver cells exhibited significant transthyretin promoter activity, no efficient viral production was detected after Ad5WS2 infection. Based on these observations, we suggest that the tumor-selective replication of Ad5WS2 may be determined using the similar mechanism proposed by O'shea. However, we could not rule out other unprecedented differences existed between tumor and normal cells that might confer to the discrepant replication ability of Ad5WS2.

Because it has been generally thought that adenovirus is unable to complete the replication cycle in normal murine tissues, such as lung and fibroblasts, most preclinical studies for replication-selective adenoviruses assess *in vivo* antitumor efficacy using human tumor xenograft models in immunodeficient mice. Giving that

antiviral immune responses have impacts on antitumor efficacy of oncolytic adenoviruses, syngeneic murine tumor models in immunocompetent mice provide more clinically relevant systems for these studies. In mice, intravenous administration of adenovirus type 5 resulted in viral replication within the liver, as evidenced by detections of viral late proteins, as well as virus particles, and a dramatic increase of viral levels 48 h after infection.[36] In mouse cell lines, it has also been shown that mouse epidermal cells are permissive for replication of human adenovirus type 5.[37] Some mouse tumor cell lines can support viral uptake, gene expression, and replication of oncolytic adenoviruses.[38] To this end, we show that not only viral early protein E1A, but also late proteins hexon and fiber were detected in Ad5WS2-infected ML-1 cells, suggesting that productive replication of Ad5WS2 occurred.

Invasion of HCC to the peritoneum causes malignant ascites, which is frequently found in patients with advanced HCC.[39] To be more clinically plausible, in our study, we systemically injected virus in mice bearing tumors. The profile of neutralization antibody was monitored before we set up the regimen. After one shoot of intraperitoneal injection, only low to moderate levels of neutralization antibody were detected against virus within two weeks (Supporting information sTable 1). Therefore, to reduce the possible effects of neutralization antibody on virus, we injected mice i.p. and i.v. with Ad5WS2 three and four times, respectively, at intervals of two days. In mice bearing ascites ML-1 tumors and orthotopic ML-1 tumors, treatment with Ad5WS2 significantly prolonged the mice survival as compared with Ad5WS1. However, Ad5WS1 and Ad5WS2 exhibited similar effect against ML-1 cells in the *in vitro* cytotoxicity analysis and mice subcutaneous tumor models. The replication rate of adenoviruses may partially account for the divergent therapeutic effect of these two viruses. In a plaque development assay in 293 cells, we found that Ad5WS1 replicate faster than Ad5WS2 (Supporting information Fig.S6). When delivered systemically, slower replication rate of Ad5WS2, may have accounted for a lower viral yield found in the mouse liver, which may result in lower hepatic toxicity. Nevertheless, the E1A protein was also detected in the liver of Ad5WS2-treated mice, albeit to much less amount, than that of Ad5WS1-treated ones. Another factor contributing to the lower toxicity of Ad5WS2 is the liver-specific transthyretin promoter that drives E1A expression. Since expression of E1A in Ad5WS1 is controlled by its native constitutive E1A promoter, Ad5WS1 would express E1A in all tissues even though efficient replication does not occur. As a result, toxicity of the transduced normal cells may occur due to E1A-induced apoptosis and other pleiotropic effects.[40] Immunological responses raised by rapid viral replication and non-specific expression of viral protein resulted in more serious acute inflammation after systemic treatment of Ad5WS1. The increased serum ALT levels in Ad5WS1-treated mice demonstrated

more serious liver toxicity than those in Ad5WS2-treated ones. These effects caused by immune responses are the reasons we do not observe cytotoxicity of Ad5WS1 against Chang liver in the *in vitro* study. Rather, immune responses also play essential roles against tumors, too. Viruses have evolved to kill cells by direct cell death machinery and fairly brisk immune responses. Endo *et al.* reported that tumor cell death caused by oncolytic adenoviruses could prime dendritic cells with tumor-associated antigens.[41] The cytotoxic T-lymphocytes then activated by these antigen-presenting cells would further eliminate the virus–infected cells. Ad5WS2 trends to replicate in liver tumor cells, which may initiate more effective antitumor immune responses in liver tumors. Therefore, through systemic administration, immune responses occurred in the Ad5WS1- and Ad5WS2- treated animals might be different and thus resulted in diverse therapeutic effect of these two viruses.

In conclusion, our *in vitro* and *in vivo* results demonstrate that transthyretin promoter-driven Ad5WS2 exhibits more potent antitumor efficacy than Ad5WS1 for the treatment of HCC in the tumor models closely resembling the clinical settings. Therefore, this study may have clinical implications in exploring E1B-55 kD-deleted adenovirus under the transcriptional control of the transthyretin promoter for the treatment of primary and metastatic HCC.

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附圖

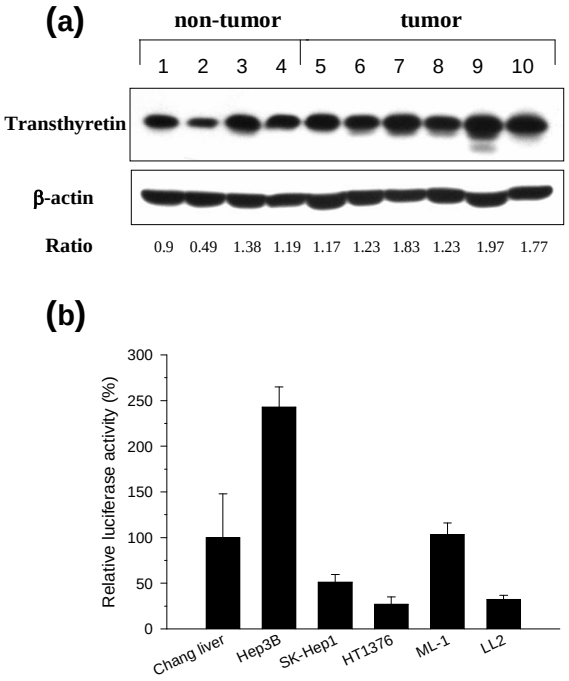


Figure 1 Detections of transthyretin protein and transcriptional activity of tranthyretin promoter in various human and murine cells. (a) Transthyretin protein was produced in the non-tumor (Lanes 1-4) and tumor (Lanes 5-10) parts of the liver from HCC patients, as determined by immunoblot analysis. Lanes 1 and 6, 3 and 9, and 4 and 7 were paired samples. Expression of β-actin served as the quantitative control. *P*=0.04 for tumor group vs non-tumor group by unpaired Student's *t* test. (b) The activity of the transthyretin promoter was higher in liver cell lines compared with non-liver cell lines. Cells were transfected with pGL-TTRp and the transfection efficiency was standardized to the cotransfected plasmid pTCYLacZ expressing β-galatosidase driven by the β-actin promoter. Relative luciferase activity was indicated by comparing each cell line with Chang liver cells. Each value represents the mean ± SD (*n*=3).

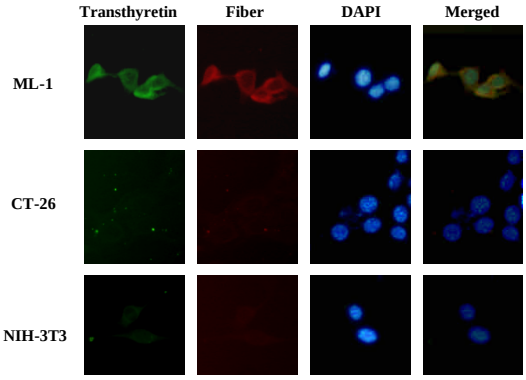


Figure 2 Detections of transthyretin and viral protein in Ad5WS2 infected cells. Cells were infected with Ad5WS2 (MOI=3) for 48 h and immunofluorescent double staining were performed with rabbit anti-transthyretin antibody by fluorescein and mouse anti-fiber antibody by Texas-Red. Nuclei were counterstained with DAPI. The merged column represents the superposition of the cells stained with anti-transthyretin and anti-fiber, and DAPI to visualize colocalization. Representative sections were shown (original magnification ×400).

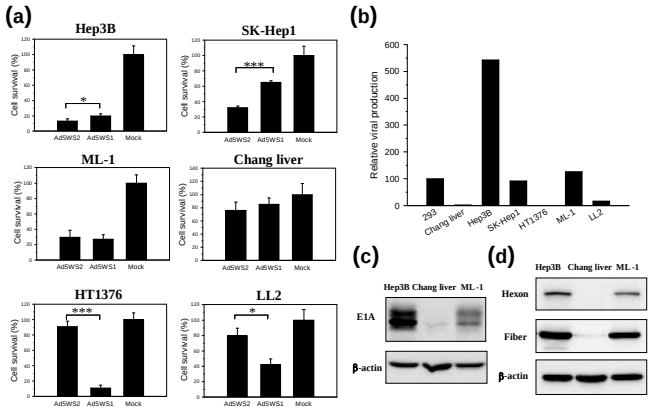


Figure 3 Selective replication of Ad5WS2 in human and murine HCC cell lines. (a) Ad5WS2 induced more cell death in human and murine HCC cells, but highly attenuated in non-HCC cells. Cells were infected with Ad5WS2 or Ad5WS1 at an MOI of 2. The viable cell numbers were determined after 3 days by trypan blue exclusion. Each value represents the mean ± SD (n=3). *, $P<0.05$; ***, $P<0.001$. (b) Human and murine HCC cells produced higher viral titers than cancer cells from non-liver origin following infection with Ad5WS2. Various cells were infected with Ad5WS2 or Ad5WT at an MOI of 10. Fifty-three hours postinfection, viral titers were determined in 293 cells by the $TCID_{50}$ method. The amount of Ad5WS2 produced was normalized against the amount of Ad5WT produced in the same cell line. Relative viral production was indicated by comparing each cell line with 293 cells. (c) Expression of adenoviral E1A protein was detected in Hep3B and ML-1 cells at 36 h postinfection with Ad5WS2 at an MOI of 10. (d) Expressions of adenoviral hexon and fiber proteins were detected in Hep3B and ML-1 cells at 48 h postinfection with Ad5WS2 at an MOI of 1.

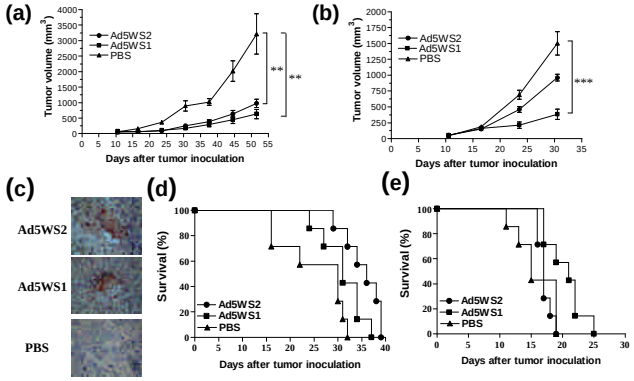


Figure 4 Antitumor effects of Ad5WS2 on subcutaneous and ascites ML-1 tumors. (a) Ad5WS2 and Ad5WS1 exhibited similar antitumor activity against ML-1 tumors. (b) Ad5WS1, but not Ad5WS2, exerted antitumor activity against LL2 tumors. ML-1 (a) and LL2 (b) cells (1×10^6) were inoculated s.c. into the flank of BALB/c and C57BL/6 at day 0, respectively. The mice were treated i.t. with Ad5WS2 or Ad5WS1 (2×10^7 PFU) every other day from day 12 to day 20. The tumor volume is presented as mean ± SE (n=8). **, $P<0.01$; ***, $P<0.001$. (c) Adenoviral hexon protein was detected in the tumor nodules from mice bearing ML-1 ascites tumors after systemic administration of Ad5WS2 or Ad5WS1. Groups of 4 mice were injected i.p. with Ad5WS2 or Ad5WS1 (5×10^7 PFU/dose), or with PBS at days 3, 5, 7, and 9, and sacrificed at day 15. Representative sections were shown (original magnification ×200). (d) Ad5WS2 was more efficacious than Ad5WS1 in enhancing the survival of mice bearing ML-1 ascites tumors. (e) Ad5WS1 but not Ad5WS2 prolonged the survival time of mice bearing LL2 ascites tumors. ML-1 (d) and LL2 (e) cells (2×10^6) were inoculated i.p. into groups of seven BALB/c and C57BL/6 mice at day 0, respectively. Mice were injected i.p. with Ad5WS2 or Ad5WS1 at a dose of 5×10^7 PFU at days 3, 5, 7, and 9. Kaplan-Meier survival curves were shown and analyzed by the log rank test. P=0.0034 for Ad5WS2 vs PBS in (d); P=0.0224 for Ad5WS1 vs PBS and P=0.0105 for Ad5WS2 vs Ad5WS1 in (e).

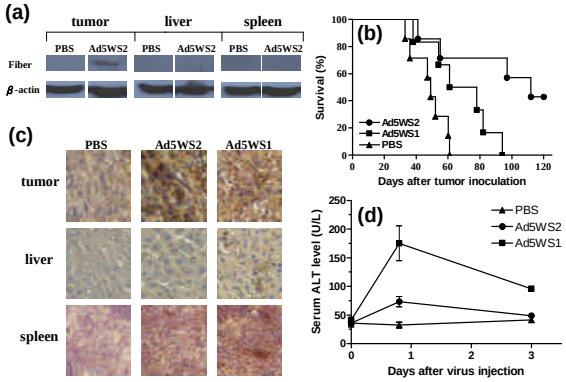


Fig. 5. Antitumor effects of Ad5WS2 on orthotopic ML-1 tumors. (a) Adenoviral fiber protein was detected in the tumor nodules from mice bearing ML-1 orthotopic tumors but not normal liver and spleen tissue after systemic administration of viruses. ML-1 cells (2×10^5) were inoculated into the left liver lobe of BALB/c mice at day 0. Groups of 4 mice bearing orthotopic ML-1 tumors were then injected i.v. with Ad5WS2 (2×10^7 PFU) or PBS at days 30, and sacrificed at day 33. Expression of β-actin served as the quantitative control. (b) Ad5WS2 exerted higher antitumor efficacy than Ad5WS1 in mice bearing orthotopic ML-1 tumors. ML-1 cells (2×10^5) were inoculated into the left liver lobe of BALB/c mice at day 0. Groups of 6-7 mice bearing orthotopic ML-1 tumors were then injected i.v. with Ad5WS2 or Ad5WS1 (2×10^7 PFU) at days 15, 17, and 19. Kaplan-Meier survival curves were shown and analyzed by the log rank test. P=0.008 for Ad5WS2 vs PBS, P=0.0305 for Ad5WS1 vs PBS, and P=0.0257 for Ad5WS2 vs Ad5WS1 in (b). (c) Adenoviral E1A protein was detected in the tumor nodules from mice bearing ML-1 orthotopic tumors but not normal liver and spleen tissue after systemic administration of viruses albeit to substantial level of E1A protein in Ad5WS1-treated group. Groups of 4 mice bearing orthotopic ML-1 tumors were injected i.v. with Ad5WS2 or Ad5WS1 (2×10^7 PFU) at days 30, and sacrificed at day 33. Representative sections were shown (original magnification ×400). (d) Mild and transient elevation of serum ALT levels were shown in ML-1 tumor-bearing mice receiving Ad5WS2. Sera were collected 19 h, as well as 3 days after virus injection and measured for ALT levels. Each value represents the mean ± SE (n=4).

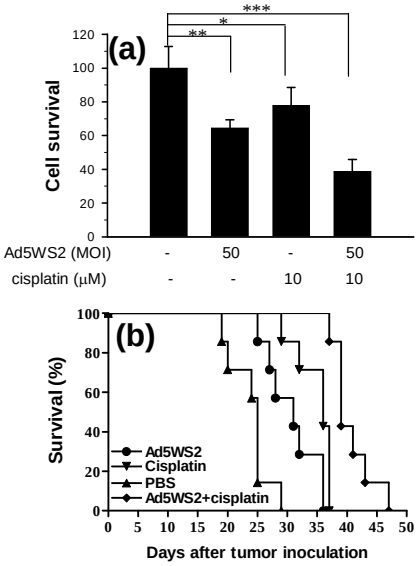
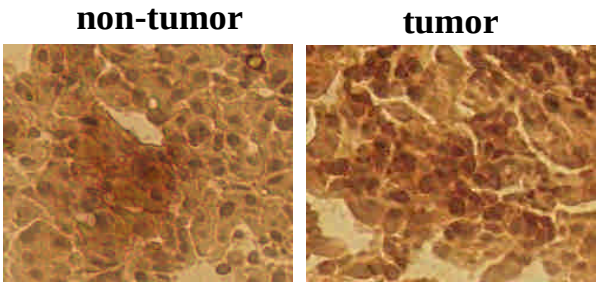
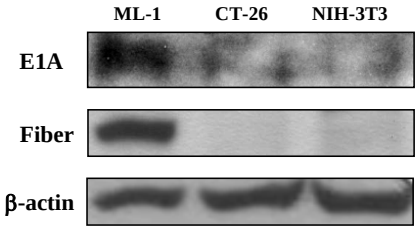


Figure 6 Antitumor effects of Ad5WS2 were enhanced in combination with cisplatin on ML-1 ascite tumor. (a) Ad5WS2 induced more cell death in the combination with cisplatin. ML-1 cells (5000 cells/well) were treated with Ad5WS2 (MOI=50) and cisplatin (10 μm) or with either treatment alone. The viable cell numbers were determined after 4 days by WST-1 assay. Each value represents the mean ± SD (n=4). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (b) Ad5WS2 in combination with cisplatin exerted higher antitumor efficacy compared with either Ad5WS2 or cisplatin treatment alone. Groups of 7 mice were inoculated i.p. with ML-1 cells (2×10^6) at day 0. For single agent treatment, mice were treated i.p. with either Ad5WS2 (5×10^7 PFU) at days 3, 5, 7, and 9 or cisplatin at days 11, 13, 15, and 17. For combination treatment, four doses Ad5WS2 (5×10^7 PFU/dose) were injected i.p. at days 3, 5, and 7, and then treated i.p. with cisplatin at days 11, 13, 15, and 17. Kaplan-Meier survival curves were shown and analyzed by the log rank test. P=0.0002 for Ad5WS2 plus cisplatin vs Ad5WS2, P=0.0011 for Ad5WS2 plus cisplatin vs cisplatin, P=0.0004 for cisplatin vs PBS, and P=0.0066 for Ad5WS2 vs PBS in (b).

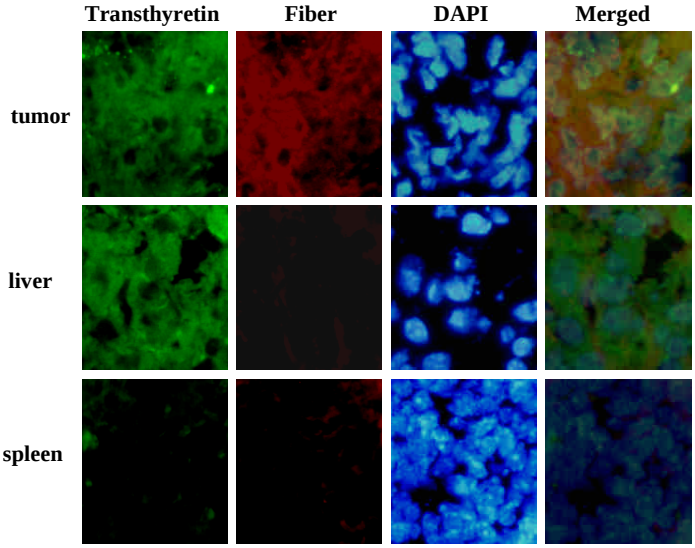
補充資料



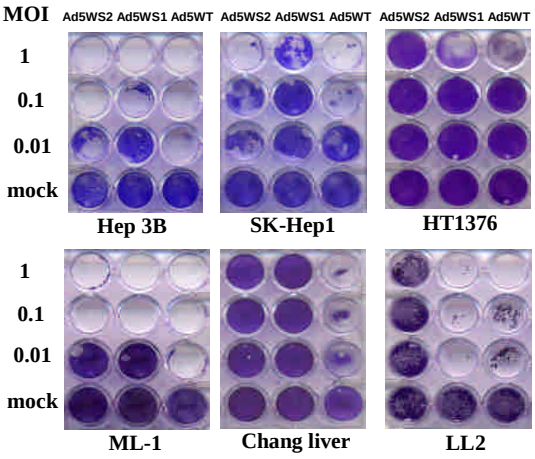
Detections of transthyretin protein in human HCC. Cytoplasmic expression of transthyretin in non-tumor and tumor parts of the liver from HCC patients was detected by immunohistochemistry (original magnification ×400).



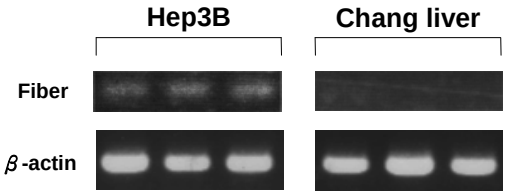
Detections of viral protein in Ad5WS2 -infected tissues. Expression of adenoviral E1A and fiber proteins were detected in ML-1 cells at 36h, 48h, respectively postinfection with Ad5WS2 at an MOI of 3, but not CT-26 and NIH-3T3 cells, indicating a restricted viral replication in liver tumor cells .(Expression of β-actin served as the quantitative control).



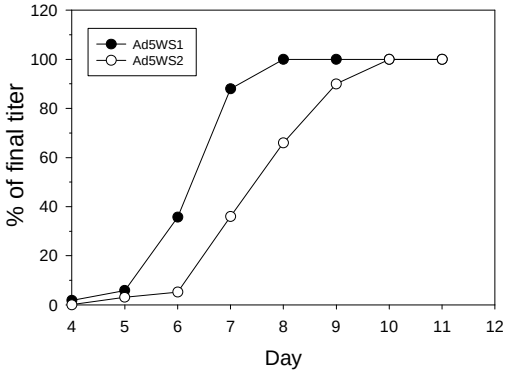
Detections of transthyretin and viral protein in Ad5WS2 infected tissues. The doubling staining was performed on tissues removed from animals after systemic administration of Ad5WS2. The result was shown above. ML-1 cells (2 $\times 10^5$) were inoculated into the left liver lobe of BALB/c mice at day 0. Groups of 4 mice bearing orthotopic ML-1 tumors were then injected i.v. with Ad5WS2 (2 $\times$ 10^7 PFU) at days 30, and sacrificed at day 33. Consistent with the *in vitro* data, the late viral protein was detected in liver tumor tissues. These data indicated Ad5WS2 could only replicate in tumor cells where transthyretin gene is active.



Cytolytic effects of Ad5WS2, Ad5WS1, or Ad5WT on various cells. CPE was observed in all the cell lines tested following Ad5WT infection, whereas it was detected in all the tumor cell lines after Ad5WS1 infection. Ad5WS2, however, only induced CPE in HCC cell lines. Cells were infected with varying doses of Ad5WS2, Ad5WS1, or Ad5WT, and monitored for CPE by crystal violet staining at 4-7 days postinfection.



Detection of fiber mRNA expression in Ad5WS2-infected Hep3B cells by RT-PCR analysis. RNA was isolated using the quickGene RNA cultured cell HC kits (FujiPhoto Film Co., Ltd. Life Science Product Division, Tokyo, Japan). RNA (1 μg) was then reverse-transcribed into cDNA according to the manufacture's instruction (ABgene, Surrey, UK). PCR was performed on the samples in triplicate with specific primers for L5-fiber [5'-CGG CCT CCG AAC GGT ACT-3'(forward) and 5'-TCT TGC GCG CTT CAT CTT G-3' (reverse)]; and for β-actin [5'-GAG AAG CTG TGC TAT GTT GCT CT-3'(forward) and 5'-ATG ATC TTG ATC TTC ATG GTG CT-3' (reverse)]. The reaction conditions were denaturing at 95℃ (30 s), annealing at 56℃ (30 s) and extension at 72℃ (1 min). The reactions were completed by a final extension at 72℃ (7 min).



Plaque development assay. Viruses were prepared with serial dilutions and visible plaques were counted every day 96 h after infection. The number of plaques on a given day was plotted as a percentage of plaques seen on the final day of the assay. Each point represents the average of duplicate determination.

sTable 5 Titer of neutralization antibody against adenoviruses in the sera after virus treatment

Treatment	neutralization antibody titer (mean ± S.D.)	
	6 days	14 days
Ad5WS2* (4)	2, 4, 4 (3.3 ± 1.2)	4, 8, 8 (5.3 ± 2.3)
PBS (4)	＜2	＜2
Preimmune (4)	＜2	＜2

*Mice (n=4) were i.p. injected with Ad5WS2 (2 x10^8 PFU) or PBS. Mice serum samples were collected before (preimmune) and after virus injection. NAB titers were analyzed by determining the ability of serum to inhibit the infection of Ad5WT to 293 cells. Various dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of antibodies preincubated with the Ad5WT (100 TCID50) for 1 h were added to 90% confluent 293 cells. Cells were then incubated for 4 days, and sera were scored positive for neutralization when the protection of cytopathic effect was ＞50%.

計畫成果自評

In this study, we constructed a liver-specific E1B-55 kD-deleted adenovirus, designated Ad5WS2, under the control of the transthyretin promoter. We have successfully demonstrated that Ad5WS2 selectively replicates and hence lysed HCC cells. Furthermore, Ad5WS2 exhibited high antitumor activity via systemic administration in an orthotopic and ascites syngeneic HCC model. Lack of viral replication in normal organs and minimal hepatic toxicity was noted after Ad5WS2 treatment. The antitumor effect of Ad5WS2 was in synergy with cisplatin in mice bearing liver tumors accompanied with malignant ascites. Therefore, Ad5WS2 represents a potentially applicable anticancer agent for the treatment of primary and metastatic HCC.

As the project we previously proposed, we have now already completed 100% of this project. The whole data was collected and submitted. It had been accepted for publication in the journal, *Cancer Science* (the forthcoming volume). We believe that these pre-clinical studies will provide the scientific foundation for future development of recombinant oncolytic adenovirus as an effective therapeutic agent for primary HCC and ascites associated HCC.

出席國際學術會議心得報告

計畫編號	NSC 95-2320-B-273-002-MY2
計畫名稱	以癌溶性病毒作肝原位癌的基因治療（第 2 年）
出國人員姓名 服務機關及職稱	謝政蓉 (中華醫事科技大學護理系副教授)
會議時間地點	11/13/2008-11/16/2008 比利時布魯日(Brugge, Belgium)
會議名稱	XVIth Annual Congress of the European Society of Gene and Cell Therapy
發表論文題目	Adenovirus-mediated kallistatin gene transfer ameliorates disease progression in a rat model of osteoarthritis induced by anterior cruciate ligament transection

一、參加會議經過

此會議今年於歐洲比利時一中世紀古城，布魯日舉行，此乃是基因治療界每年主要的盛會之一，各地從事相關研究學者齊聚於此發表每年的研究成果。在為期四天的議程之中，共有一百一十場的口頭研究發表與兩百八十個壁報研究發表以及四十場的座談會，於一大會議場地，不同的會議廳，在四天之中同時進行。針對載體 (vectors) 的研究與改良、臨床治療先天遺傳性與後天性疾病的進展、幹細胞 (stem cells) 技術的研發、以及基因治療所面臨醫學倫理的問題等多方面的研究，作成果發表。與會人士可以選擇個人有興趣的主題參加。我除觀摩他人的研究成果，亦參加的壁報發表的部份，針對所研究的主題：利用腺病毒攜帶抗發炎因子 kallistatin 進行退化性關節炎的基因治療作研究成果的發表 (請見附件內容摘要部分)。

二、 與會心得

由這兩年參加此會議的經驗顯示，基因移轉 (gene transfer) 的技術正快速的進步中；而臨床的實驗結果證明，利用基因治療的方法可實際運用於許多疾病之中，包括：腫瘤、先天性免疫缺失 (inherited immune deficiencies)、骨骼肌肉疾病 (musculoskeletal)、貧血 (hemophilia) 、神經性的疾患等，如帕金森氏症 (Parkinson's disease) 。在此次所看到的研究發表中，除了腫瘤與先天代謝性疾病的基因治療仍佔研究的一席之地外，也看到越來越多的科學家投身於利用 RNA 干擾技術 (RNA interference) 作疾病的治療。此外，利用幹細胞技術修改過的 T 細胞來治療先天性與後天性的遺傳疾病也有增加的趨勢。吾人能預見這一區塊在基因治療上頗具潛力，必將是未來研究的主流之一。出席這次的會議，提供我們得以與國際間此方面的翹楚面對面討論的機會，獲益良多。此外，利用壁報發表的機會得以認識其他研究相關的人，針對腺病毒載體和關節炎治療分享實驗和研究的心得，實是一種非常難得的機會，除了能提供研究方面新的思維之外，個人認為藉此機會能增加本國此方面研究在國際之間的交流。感謝國科會能補助吾等研究領

域的人能出國與人相互觀摩切磋，交換心得，所獲所得非僅由網路、期刊單項取得訊息可比擬。

附件

ESGCT 2008 POSTER PRESENTATIONS (published in *Human Gene Therapy* 19: (10), p1174, 2008)

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Adenovirus-mediated kallistatin gene transfer ameliorates disease progression in a rat model of osteoarthritis induced by anterior cruciate ligament transection

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Objective: In osteoarthritis (OA), inflammation and apoptosis are two important factors contributing to disease progression. As kallistatin can suppress inflammatory responses and reduce cell apoptosis, we investigated the therapeutic effect of kallistatin gene transfer in the rat model of OA by anterior cruciate ligament-transection (ACLT).

Methods: OA was induced in Wistar rats by ACLT in the knee of one hindlimb. Adenoviral vector encoding human kallistatin (AdHKBP) was injected intraarticularly into the knee joints after ACLT. The viral effect on tissues was evaluated. The transgene expression and inflammatory responses were determined by immunoblot analysis, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. The apoptosis of chondrocytes was quantified by TUNEL assay. The effects of kallistatin in combination with hyaluronic acid (HA) on the medial femoral condyles and synovia were also assessed histologically.

Results: The expression of human kallistatin after intraarticular injection was identified. Kallistatin gene transfer reduced the levels of interleukin-1β (IL-1β) and tumor necorsis factor-α (TNF-α) in joints. Examination of gross morphology revealed that rats treated with AdHKBP had reduced severity of OA compared with the control rats treated with adenoviral vector encoding green fluorescent protein (AdGFP). The protective effect of kallistatin on cartilage was accompanied by a decrease in apoptotic cells. Intraarticular administration of AdHKBP, when in conjunction with HA, significantly improved histologic scores in the knee joint.

Conclusions: Local administration of adenoviral vectors encoding kallistatin significantly suppressed OA progression, accompanied by reduction of inflammatory response and apoptosis. Thus, kallistatin gene therapy may be a potential treatment for OA.